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Filtrable Forms of Lactobacilli.

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The existence of filtrable forms in lactobacilli has been noted by few authors. Rudakov (1), observing the development of Bact. casei and Bact. bulgaricum, points out that these bacteria, side by side with the microscopically visible gonidia that enable them to propagate, also produce microscopically invisible filtrable forms that, the author presumes, also take part in the reproduction process of the indicated bacteria.

What distinguishes these filtrable forms from the filtrable forms of other bacteria is that the forms received by the author quickly germinate upon transfer to nutrient media and change into normal bacilli.

Similar results were received by Skalon (2). But, in Skalon's experiments the filtrable forms of lactobacilli formed irregularly and developed very slowly. The cultures growing from these filtrable forms possessed weak activity and differed morphologically from the original. In Skalon's work one question remained unexplained: are the filtrable forms received by him identical to the gonidia described by Rudakov, or do these forms have no connection with the culture's reproductive process.

As is well known, the lactobacillus, Bact. delbrueckii, reproduces by fission, with which the septum within the cells is not always formed in the center of the bacillus; it is sometimes formed nearer to the end; and as a result there is observed the so-called "unequal division of cells."

Observation of the development of Bact. delbrueckii in a hanging drop have shown that apart from the reproduction by the usual cell division, there are, in the first hours after a culture's planting in either liquid or solid media, small buds formed on the ends of some of the bacilli (see figs. 1 and 3). The formation of these buds, as already discussed, is due to the development of an internal septum in the bacilli. In a liquid medium these buds, in the majority of cases without separating from the maternal cell, increase in size and change into normal bacilli; in some cases the buds, having formed, separate from the maternal cell. In our observation of the hanging drop we were unable to trace the fate of the buds that broke away; because, under the influence of the Brownian movement they disappeared from the field of vision. But from our observation with an electron microscope

one can assume that the buds that split away will later germinate. With an inspection of the preparations made from cultures in their later hours of development the buds are almost not detected.

On solid media (in a hanging drop) we detected no separation of buds from the maternal cell. In elongating they change into normal bacilli. Quite often, particularly on agar, the bacilli do not disjoint and then long threads are formed (fig. 2).

Due to this it was important to detect whether a Bact. delbrueckii culture possesses smaller bacilli that are invisible to an optical microscope, and what their role is in the growth of the given culture. From the literature it is known that the filtrable forms of bacteria result both from action on the bacteria by various factors that destroy the cellular membrane, and from their natural aging. There are also indications that filtrable forms are produced even in very young cultures.

We conducted our experiments principally with old cultures (two weeks and older), without resorting to any sort of external effects on the cells.

We used nutrient media favorable to the growth of the original culture:

1. Beer wort of 7° and 10° Balg. with a 4 % yeast autolysate.

2. Beer wort with a pellet containing a 4 % yeast autolysate.

3. A semisynthetic sucrose medium consisting of an extract from 6 % malt growths + 2 % sucrose or glucose + 0.1 % phosphate.

4. The same media with additions of 0.1 and 1 % agar.

In the above media the pH ranged from 5 to 6.5.

As a control we used media that refused growth to the original culture: meat-peptone broth, meat-peptone agar, and milk.

The filtration of the culture was made through Seitz filters, containing SF and EK brand asbestos pads, into a receptacle, from where the filtrate was transferred by pipette into test tubes containing the different nutrient media.

Because of the possibility of air contamination in such a transfer we used this method only in the experiments with nurses (the Suknev-Volferts's or Ermol'eva "nursing method" - Translator's note), or with the fermented media; and, in the other cases the receptacle was connected to test tubes or flasks (containing sterile nutrient media) by means of glass T-pipes. In this manner the filtrate from the receptacle fell directly into sterile media; and, additionally important, we had several parallel test tubes or flasks containing the same filtrate.

The filtration was accomplished within 30-40 minutes by means of a water-jet pump with a slight vacuum.

The test tubes and flasks containing the nutrient media and filtrates were placed into a thermostat at 40°C and kept there until a sediment appeared, and then were retained at room temperature. The control test tubes were placed into a thermostat at 40 and 37°C.

In all, 60 filtrations were made from the original culture; of these, 10 filtrations were of a young culture (4 hours after inoculation), and 50 of an old. In addition, 10 filtrations of sterile tap water were made and the filtrates inoculated into sterile nutrient media.

Many investigators explain the absence, or rare formation, of filtrable forms by the forms' retention on the filter. In view of this, in some of the experiments, simultaneously with the filtration through the Seitz filters, the cultural fluid was inoculated by the dilution method (after a preliminary calculation of the bacterial count in a Tom chamber) into a series of test tubes containing a sterile nutrient medium.

For regeneration of the filtrable forms into the original culture we adopted several methods that have been described in the literature:

1. The nursing method in Petri dishes, according to Suknev and Vol'ferts.

2. The nursing method in a liquid medium, according to Ermol'eva.

3. Fermented media, according to the Kalina method.

4. Systematic transplants into liquid, semiliquid, and solid nutrient media.

The experiments with nurses.

As nurses, we utilized the lactic yeast, Sacch. lactis. This was deposited in rings on a wort agar that had been pre-inoculated with a filtrate of the original culture. The Petri dishes containing the filtrate and nurses were placed into a thermostat at 40°C. Other than yeast, no other colony formations were detected in any of the 10 experiments staged.

Also, in the inoculations from these dishes' different areas into liquid, and solid nutrient media, only yeast growth was detected. Moreover, after prolonged retention in a thermostat the dishes were intergrown with air microflora. Consequently, subsequent experiments on growing filtrable forms by the nursing method were staged in a liquid medium. In these experiments 10 test tubes containing beer wort were inoculated with the yeast, Sacch. lactis; the filtrate of the old, or young, culture was added to 5 of the test tubes; the remaining 5 test tubes, without the filtrate, served as a control on the nurses' purity. Test tubes containing the filtrate alone and testtubes with the filtrate + meat-peptone broth served as a control on the filtrate's purity. Upon completion of fermentation in the test tubes the yeasts settled to the bottom, and the medium above the sediment became clear.

In one of the experiments, after ten days, turbidity was noted in the test tubes containing the nurses and the filtrate. While in the test tubes

containing the nurses alone, there was no turbidity. By making a series of transplants into beer wort from the upper part of the "yeast + filtrate" test tubes, without touching the yeast sedimentation, and by keeping these test tubes in a thermostat at 40°C, we succeeded in eliminating the yeasts. With this the turbidity in the test tubes remained, but became significantly weaker. The transplants from these test tubes onto wort agar, meat-peptone agar, and broth would not grow.

Beneath a microscope a small quantity of oval-form bodies, approximately 1 micron in size, were detected. At first the weak turbidity was formed on the tenth 24-hour period, or later. Subsequent transplants of these forms into a beer wort- pellet induced them to form a strong "veil" within three days. The medium's pH lowered insignificantly in the first transplants, but after several transplants the pH began to decrease significantly; but, still, its change was weaker than that of the original culture.

The external appearance and distribution of the new forms also changed, according to their degree of development. At the beginning of the cultivation the oval-form cells were distributed in the field of vision singly, in pairs, or in clusters. Later, after several transplants, small chains consisting of several cells began to appear, and towards the end small rods were detected, size 1 x 0.5 microns and larger. It was possible to discover the oval-form cells and the rods in the same chain (figs. 4 and 5).

. The culture that was received grows well on beer wort with a pellet, on a sucrose medium, and on a sucrose medium containing 0.1 % agar; on a sucrose slant agar it forms colorless, very small oval-form colonies; within the agar it forms colonies in the form of lenticels. The culture absolutely refuses to grow on meat-peptone broth, on meat-peptone agar with sugar supplement, on potato media, and on milk.

Because only one of the ten experiments in this series produced microforms from the filtrate of an old culture, and because these microforms would not revert to the original culture, their appearance raised a doubt as to their origin. However, the supposition that this culture could have come from the air can be refuted - so it seems to us - by the following facts.

1. The turbidity appeared in five parallel test tubes, and the microscopic picture was identical in all.

2. The test tubes containing the filtrate alone and those containing the filtrate + meat-peptone broth were clear, and seedings from them would not grow on meat-peptone broth, on agar, nor on milk.

3. In the series of experiments (10) with the tap water's filtration, on the same media, such forms were not detected. With a contamination by common bacteria and fungi from the air, growth is very quickly detected.

The supposition that the filtrable forms of other lactic acid bacteria could have entered with the yeast used by us as nurses is dismissed by the fact that in the test tubes containing the yeast without the filtrate such forms were not detected. Nevertheless, it was necessary to determine whether such forms will be produced without the nurses' participation.

In relation to this the next series of ten experiments was conducted with fermented media. As a fermented medium we took a suspension of a four-hour culture of Bact. delbrueckii on beer wort. One portion of the suspension was sterilized at 100°C for 40 minutes (medium A); the other was filtered through a Seitz filter (medium B). The old culture's filtrate, which was to be tested, was introduced into the test tubes containing the above mentioned media. The control on the sterility of the fermented medium B was the test tubes containing the fermented medium alone, and those with the fermented medium containing meat-peptone broth.

In five experiments, after 10 to 21 days, a very weak turbidity appeared in the test tubes containing the fermented medium "B + filtrate." Once formed, the turbidity did not increase; the medium's pH remained unaltered. Three weeks after the turbidity's appearance the first seeding was made from the test tubes onto the same media. The first transplant showed that the fermented media prepared by us was worthless. Turbidity was absent in all test tubes. Nor was it detected in medium A. The test tubes containing the fermented medium without the filtrate and those with the filtrate + meat-peptone broth remained clear. The seedings from these test tubes gave no growth. There was still a possibility of receiving positive results by varying the fermented medium's preparation; for example, by selecting a culture of a different age, or by filtering for different periods of time, etc.

But in this experimental series the initial culture's filtrate was inoculated also into a beer wort-pellet medium, in addition to the fermented media. Upon detecting turbidity in the "beer wort-pellet with yeast autolysate + filtrate" test tubes, and after the turbidity formed again in subsequent transplants, we decided to discontinue use of the fermented media; although, we do not deny that in principle the fermented media can give positive results. In the beer wort with the yeast autolysate + filtrate " medium in this series, consisting of 10 experiments, a weak turbidity was was detected in six. Just as in the experiments with the fermented medium, in three of the experiments on the beer wort-pellet the turbidity was unstable, and after two transplants it refused to form: in the other three experiments we succeeded in producing a clearly transplantable turbidity. The same formations of the different sized oval forms were detected under the microscope as in the experiments with the nurses. The forms that were detected stain with methylene blue and with the other aniline dyes, although not in equal intensity. With each transplant into the beer wort-pellet, transplants were also made onto meat-peptone agar and wort agar, but growth on the agar was not detected.

Because the forms that were visible to us did not change externally in subsequent transplants, we decided to subject them to the action of weak acids and alkalis. We had in mind that if the forms that we had discovered were protein particles, then after hydrolysis they must either dissolve or alter their form. However, after hydrolysis we discovered by microscope a yet clearer image of the oval forms. The resultant forms resembled the initial stage in the formation of the microforms in the experiments with the nurses.

In view of this, 20 more filtrations were made of an old culture. In this series, in five experiments in beer wort-pellet with yeast autolysate, a weak but constantly transplantable turbidity was detected; and in two experiments the turbidity ceased to form after three transplants. In a microscopic examination the same bodies, with the oval form, were detected. Transplants into a liquid sucrose agar (0.1 %) produced a visible growth in some cases, but further transplants into this medium did not always give this.

In this same series, in five experiments, simultaneously with the filtration the original cultural fluid was introduced into nutrient media by the dilution method. In the test tubes with small dilutions, to 10^{-4}, the culture's growth was detected on the following day. In the test tubes with a dilution of 10^{-5} growth appeared within 48 hours, whereupon it was not in all cases parallel. The remaining test tubes containing further dilution of the cultural liquid remained clear over a one-month period. After a month, in the test tubes with a dilution of 10^{-6} (in which growth had at first been undetected) and in the test tubes with a dilution of 10^{-7} there was noted a very weak turbidity, which in two experiments was also transplanted in sequent subinoculations. In a microscopic examination cells of an oval form were discovered. In these two experiments, in the test tubes containing "Seitz filtrate + beer wort," which was also introduced by the dilution method, turbidity was formed at approximately the same time in the test tubes into which 1-0.5 ml of the filtrate had been added. With a lesser quantity of the filtrate in the test tubes turbidity was not detected.

The experiment's results indicated that under the condition of the given experiments, filtration inhibited the forms that afterward gave a transplantable turbidity. However, the filtrable forms' irregular formation cannot be explained by their entrapment during filtration alone. Evidently these forms are not all equally viable.

Thus, microforms were received after the cultural fluid's filtration in nine of the sixty experiments (table 2).

In one case, after systematic transplants into liquid and semiliquid media, we were able to raise the microforms out of an "inactive condition" - they were a bacterial culture that formed acid with almost the same activity as in the original culture. However, by their external appearance the culture differed from the original. In the other eight cultures we succeeded in expediting the appearance of a visible growth by growing them under anaerobic conditions and by systematic transplants into semiliquid media (particularly on a medium from an yeast autolysate). In a microscope's field of vision thereappeared, together with the oval-form cells, short rods of about one micron in length. But in these cultures the capability to produce acid has not, as yet, been detected. An analogous picture was also observed with two cultures that were received in the experiments without filtration.

Are these microforms stages that enter into the normal cycle of the original culture's development? To this question we must answer negatively, regardless that the filtrable forms in our experiments were received

without any abiological effects on the cells:

1. Because the "old cultures," due to the presence of exchange products, are also unrepresentative of normal conditions for showing the life cycle.

2. The forms that were received germinate very slowly, do not form acid, and their development into cells is observed in media with a pH no lower than 5. In laboratory and industrial conditions the original culture's development cycle occurs quickly with an energetic production of acids. Consequently the filtrable forms simply cannot succeed in converting into normal cells.

What are these microforms?

At first we presumed that these forms were nothing more than buds that had not had time to germinate into bacilli and had been passed through the filter. But experiments with a refiltration of these microforms showed that they originate, evidently, from more minute forms that are invisible to an optical microscope.

Observations with an electron microscope show that together with the forms that are visible to an optical microscope there are also more minute forms, in great quantity, that form long chains. Observed also were rods of different sizes (figs. 6 and 7).

On the basis of our observation one can surmise that, developing under adverse conditions, these forms exist in an inactive condition until they attain favorable conditions for their further development, wherein they may germinate into normal cells of the given species.

The microforms' capacities to grow, to increase in number, and to change morphologically can be called a special development cycle intrinsic to the given forms; this cycle, however, does not enter into the ordinary development cycle of the original bacterial culture in its normal conditions of existence. It is possible that in this sense they may be important for preservation of the species in natural conditions.

Because the microforms received by us have not, as yet, changed into the original form, we have no basis to reject a different viewpoint concerning the nature of the filtrable forms.

It is possible that the latter are degenerative forms that were developed under the influence of adverse conditions. Owing to their weak viability they cannot change into normal bacilli and, consequently, can have no material importance for the culture's normal development. Although the Bact. delbrueckii culture was carefully checked for purity prior to the experiments, one can surmise that the forms received from the filtrate were coexisting with the original culture, because an isolation from a single cell of the latter was not made.

Further investigations will show which of the above three viewpoints to be more correct.

Conclusions

1. Out of 60 filtrations of an old and a young culture, we received in nine experiments filtrable forms of Bact. delbrueckii that appeared as minute cells, primarily of an oval form.

2. One culture, which had been received by use of the nursing method in a liquid medium, after a series of transplants, began to evolve faster, to produce acid, and, simultaneously with this, to change morphologically. That is, together with the cells of the oval form there were also small rods observed.

3. Eight cultures were received through the use of systematic transplants into liquid and solid media; of these eight cultures, one was received through the filtration of a young original culture, and seven were received through the filtration of an old one.

4. In two of the five experiments similar forms were received even without filtration (with the introduction of the old culture into nutrient media by the dilution method).

5. The cultures that were received through systematic transplanting greatly resemble the culture received with the nurses in the liquid medium. They also change in external appearance, but, as yet, do not form acids.

6. An inspection of these cultures with an electron microscope enables us to draw the conclusion that the increase in the number of oval-form cells occurs from more minute cells that are invisible to an ordinary microscope.

Literature

1. Rudakov, K. I.: The development cycle of lactobacilli. Mikrobiologiya, 2: 1: 1933.

2. Skalon, I. S.: Morphological and physiological changes of lactic bacteria due to their development. Mikrobiologiya, 8: 6: 1939.

Translator's note: Of the 7 illustrations included in this article only Nos. 1 and 2 were available to the translator.

Table 1

The culture isolated from the filtrate.		The original culture.	
pH after 24 hours	pH after 48 hours	pH after 24 hours	pH after 48 hours
4.01	3.76	3.25	2.95
4.06	3.71	3.30	2.98
3.91	3.78	3.27	2.96
3.84	3.76	3.24	2.94
3.81	3.75	3.24	2.95
3.79	3.63	3.26	2.93

Table 2

Data about the different methods of regenerating the filtrable forms of Bact. delbrueckii.

Number of filtrations of an old culture	Number of filtrations of a young culture	Method of Regeneration	Number of positive tests filtrate of old culture	Number of positive tests filtrate of young culture
10	-	Nurses on Petri dishes.	-	-
10	-	Nurses in liquid media.	1	-
10	10	Fermented media and transplants into beer wort-pellet with a yeast autolysate.....	2	1
20	-	Transplants from liquid media to liquid and solid.	5	-
The experiments without filtration				
5	-	Transplants from liquid media to liquid and solid.	2	-

Fig. 1. The original culture of Bact. delbrueckii on beer wort with pellet.
Fig. 2. The original culture of Bact. delbrueckii on malt agar.

(Illustration not available)

Fig. 3. Formation of minute buds in the original culture. Electronoscopic photos. Magnified 8,000x

(Illustration not available)

Fig. 4. Appearance of the culture received from the filtrate with the nurses in a liquid medium. Electronoscopic photos. Magnified 8,000x

(Illustration not available)

Fig. 5. Appearance of the culture received from the filtrate with the nurses in a liquid medium. Magnified 1,000x

(Illustrations not available)

Figs. 6 and 7. Appearance of the microforms that were received from the filtrates. Electronoscopic photos. Magnified 20,000x